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Serological Analysis of Species Specificity in the High Mobility Group Chromosomal Proteins*

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The non-histone chromosomal protein of the high mobility group (HMG-1) present in mouse liver was purified to homogeneity. Antibodies against this protein as well as pure HMG-1 derived from calf thymus and HMG-E purified from duck erythrocytes were elicited in rabbits. The interaction between the antibodies and the immunogens was measured by passive hemoagglutination and by quantitative microcomplement fixation.

Quantitative microcomplement fixation assays revealed that the immunological distance between HMG-1 from calf thymus and HMG-1 from mouse liver and duck erythrocytes was 15. This corresponds to 3% sequence differences. It was estimated that amino acid substitution occurred at about seven positions in the polypeptide chain. Thus, HMG-1 proteins display remarkable evolutionary conservation in their primary sequence, similar to that displayed by histones H4 and H3, suggesting that their biological function is dependent on stringent structural requirements. HMG-E protein is significantly different from both HMG-1 and HMG-2 derived from calf thymus. As such, it is a protein unique to avian erythrocytes.

Chromatins isolated from various sources contain a class of nonhistone proteins known as the high mobility group chromosomal proteins (HMG) (1). Proteins contained in this group have been fractionated to homogeneity and characterized in terms of behavior in polyacrylamide gel electrophoresis, molecular weight, and amino acid composition. The complete primary structure of HMG-17 and the partial amino acid sequences of HMG[1]-1 and HMG-2 isolated from calf thymus have been determined (2, 3). All tissues so far examined (4) contain a group of chromosomal proteins with properties similar to those described for the proteins isolated from calf thymus. However, tissue-specific HMG has been described for the trout testis (5) and avian erythrocytes (4, 6). Since these proteins are present in approximately 10^6 molecular copies per calf thymus nucleus and since they are also present in inactive cells such as chicken (7) and duck erythrocytes (4, 6), it has been suggested that they do not function as primary regulatory proteins. Their role, however, may be that of permanently altering the structure of that portion of the chromatin where they are bound. Indeed, it has been shown that they can be selectively released by a limited DNase I digestion of chromatin (6, 8) under conditions which are known preferentially to digest genes which are active or genes which have a previous history of activation (9, 10).

Elucidation of the role of HMG proteins in cellular processes requires determination of the species and organ specificity of these proteins. Serological techniques provide a convenient way to assess differences in the primary structure of related proteins (11-13). The availability of purified, well characterized HMG proteins makes it possible to elicit antibodies suitable for such studies. Indeed, antisera to proteins HMG-1 and HMG-2 purified from calf thymus have been used to assess the sequence relationships among the six major nonhistone proteins present in that tissue (14). In the present report, we study in detail the immunological relatedness of protein HMG-1 purified from chromatins obtained from calf thymus, mouse liver, and duck erythrocytes. In addition, we estimate the sequence relationship between the HMG-1 proteins and the tissue-specific HMG-E obtained from duck erythrocytes.

MATERIALS AND METHODS

HMG proteins from calf thymus and duck erythrocytes have been extracted and purified essentially by the technique originally described by Goodwin *et al.* (1). The characterization of these proteins has been previously reported (4). Highly purified mouse liver nuclei (15) were used as the source of mouse HMG. The nuclei were extracted with 5% perchloric acid and the HMG proteins were separated from H1 histones by selective precipitation with acetone (16). The HMG fraction from mouse liver was further fractionated by the procedures described before (1, 4).

Immunization Procedures—Male white New Zealand rabbits weighing approximately 3 kg were used. Immunization was performed by injecting in multiple (10 to 30) intradermal sites a total of 200 μg of protein dissolved in 0.5 ml of 10 mM Tris-HCl, pH 8.8, and emulsified with an equal volume of Freund's adjuvant. The same dosage and procedure was repeated for secondary stimulation 1 month after the primary and a booster was given 15 days after the secondary stimulus with 80 to 100 μg of immunogen in the same volume.

Bleedings were taken 2 weeks after the secondary immunization and 8 days after the booster.

Passive Hemoagglutination—The antisera titer was determined with the passive hemagglutination technique as follows. Sheep red blood cells were washed three times in 0.14 M NaCl, 10 mM Na/

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[1] The abbreviations used are: HMG, high mobility group chromosomal proteins; NaCl/P_i, 0.14 M NaCl, 0.01 M sodium phosphate buffer, pH 6.8; SDS, sodium dodecyl sulfate.

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phosphate, pH 7.2, ($NaCl/P_i$), diluted to 2.5% (v/v) with $NaCl/P_i$ and incubated for 30 min at room temperature with an equal volume of tannic acid in $NaCl/P_i$ (1/20,000, w/v). The cells were washed with $NaCl/P_i$ and a 2.5% (v/v) cellular suspension in $NaCl/P_i$ was incubated with an equal volume of antigen solution in $NaCl/P_i$ (200 μg/ml) for 30 min. The cells were then washed with $NaCl/P_i$ containing 1% of heat-inactivated normal rabbit serum to prevent nonspecific agglutination and resuspended at a final concentration of 1.25% (v/v) in the same medium.

Fifty microliters of heat-inactivated antisera in serial dilutions were added in a microtiter plate to 50 μl of sensitized sheep red blood cells and the plates were incubated for 2 h at 4°C. Controls were made with normal rabbit serum, with tannic acid-treated but unsensitized red blood cells and with normal red blood cells. All the control tests were negative.

Microcomplement Fixation Technique—Quantitative microcomplement fixation tests were performed according to Wassermann and Levine (17) with some modifications (14).

RESULTS

HMG Proteins—Criteria for the purity of the HMG proteins prepared from duck erythrocytes and from calf thymus have been presented elsewhere (4). HMG-1 protein obtained from mouse liver is compared to the other HMG proteins in terms of its electrophoretic mobility in SDS-polyacrylamide gel in Fig. 1. It can be seen that it migrates as a single band in the same position as HMG-1 obtained from calf thymus and duck erythrocytes. The amino acid composition of HMG-1 obtained from mouse liver is very similar to that of the homologous protein obtained from calf thymus (Table I). HMG-2 from calf thymus migrates slightly ahead of the HMG-1 proteins and is distinct from the tissue-specific HMG-E.

Characterization of Antisera—Antisera against HMG-1 from calf thymus, HMG-1 from mouse liver, and HMG-E from duck erythrocytes were tested against the homologous antigen by the passive hemagglutination test. It can be seen from Table II that all rabbits injected with the various antigens produced antibodies, although the response varied from rabbit to rabbit.

The specificity of all antisera was also confirmed by testing them against H1 and H5 histones. No agglutination was detected when red blood cells were coated with these histones even when the sera were used at full strength. However, agglutination was observed when cells coated with H1 histone were incubated with anti-H1 histone antibody.[2]

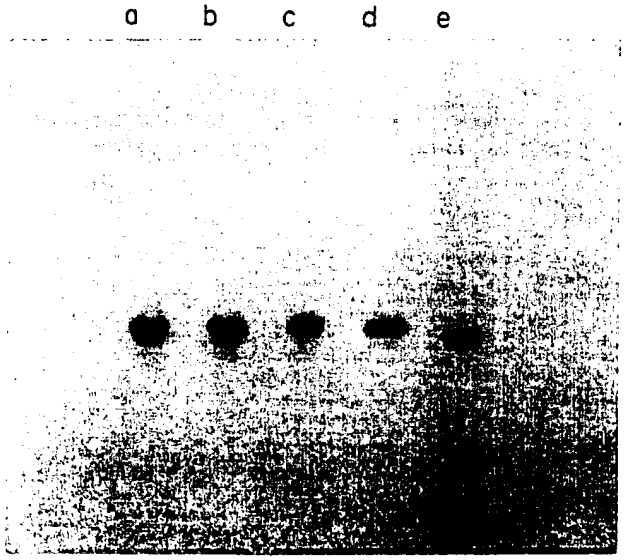

FIG. 1. SDS-polyacrylamide gel electrophoresis of purified HMG proteins. *a*, HMG-1 calf thymus; *b*, HMG-1 mouse liver; *c*, HMG-1 duck erythrocytes; *d*, HMG-2 calf thymus; and *e*, HMG-E duck erythrocytes. Migration is from *top* to *bottom*.

TABLE I

Amino acid composition of the HMG-1 from calf thymus and mouse liver

Amino acid	Calf thymus HMG-1	Mouse liver HMG-1
	M%	
Lysine	17.8	17.5
Histidine	1.2	1.2
Arginine	3.8	3.5
Aspartic acid	11.6	11.9
Threonine	3.1	3.4
Serine	5.9	6.2
Glutamic acid	20.7	20.9
Proline	7.6	7.2
Glycine	6.2	7.0
Alanine	9.5	8.5
Cysteine	N.D.[a]	N.D.[a]
Valine	2.6	2.8
Methionine	N.D.	N.D.
Isoleucine	2.1	1.7
Leucine	3.1	3.2
Tyrosine	1.7	1.7
Phenylalanine	3.1	3.3

[a] N.D., not determined.

TABLE II

Titers of antisera determined by passive hemagglutination

Rabbit immunized with	Antigen				
	HMG-1 calf thymus	HMG-1 mouse liver	HMG-E duck erythrocytes	Histone H1	Histone H5
Calf thymus HMG-1	1:1280			—[a]	—[a]
Calf thymus HMG-1	1:640			—[a]	—[a]
Mouse liver HMG-1		1:1280		—[a]	—[a]
Mouse liver HMG-1		1:1280		—[a]	—[a]
HMG-E			1:1280	—[a]	—[a]
HMG-E			1:640	—[a]	—[a]
H1				1:2560	—[a]

[a] No reaction.

Each of the antisera showed strong complement fixation when reacted with the homologous antigen. A typical series of complement fixation curves obtained with serial dilutions of anti-HMG-E is presented in Fig. 2. It can be seen that the sera at dilution of 1:3200 still gave a strong positive reaction. As previously noted with anti-HMG-1 (14), the complement fixation curves seem to be bimodal. Two different points of maximal fixation are obtained, one in the range of 0.06 to 0.12 μg of HMG-E and another at amounts higher than 1 μg of antigen. The antigen by itself is not anticomplementary. As previously suggested (14), it is possible that the antisera recognizes different molecular species of the HMG protein.

Very similar results were obtained when the antisera elicited by HMG-1 purified either from mouse liver or calf thymus were tested with the respective homologous antigen. In each case, strong, bimodal complement fixation curves were obtained. The antisera elicited against protein HMG-1 obtained from calf thymus are not the same antisera as used in a previous study (14). A different preparation of HMG-1 was used to immunize a new set of rabbits at The Rockefeller University. The availability of antisera prepared in different laboratories provided an opportunity to determine the degree to which the antisera obtained are dependent on minor experimental procedures. The antisera were, therefore, cross-tested,

[2] Anti-H1 antiserum was a gift of Dr. H. P. Hoffmann, The Rockefeller University, New York.

i.e., HMG-1 prepared in one laboratory against anti-HMG-1 prepared in another. The results (not shown) reveal complete cross-reaction between the two antisera and the two antigens.

All of the sera were also tested by microcomplement fixation against histones H2b and H1. No reaction was observed, confirming the results obtained by the passive hemagglutination technique. All these results suggest that the sera are specific for the homologous HMG proteins although, as will be presented later, all the HMG-1 proteins display strong immunological homologies.

Antigenic Similarities of HMG Proteins—The antisera were used to study the specificities of HMG-1 proteins derived from different sources by the approach used for other proteins (13, 14). The various anti-HMG sera at several dilutions were reacted with various concentrations of each of the HMG proteins tested. The maximum percentage of complement fixation obtained in each test was plotted against the antibody concentration as shown in Fig. 3, *A* to *C*. The data clearly indicate that the HMG-1 proteins immunologically are closely related. HMG-E, however, seems somewhat different. From these plots, it is possible to calculate the index of dissimilarity between two proteins, that is, the ratio of serum dilution that gives 50% maximal complement fixation with the heterologous HMG protein. The logarithm of the index of dissimilarity multiplied by 100 equals the immunological distance, which is linearly related to the percentage of sequence differences between related proteins (11, 12). The immunological distances between the various HMG proteins determined with the various antisera are tabulated in Table III.

As pointed out by Prager and Wilson (18), in an ideal system, the differences between two proteins, X and Y, should be the same regardless of whether determined with antibodies to X or antibodies elicited by Y. Thus, the reciprocity between the two measurements should be as close to 1.0 as possible. The data presented in Table III indicate that, in most cases, this is indeed so. Thus, the immunological distance between HMG-1 obtained from calf thymus and mouse liver is 15 regardless of whether it was measured with anti-mouse or anti-calf thymus HMG-1. The distance between HMG-1 obtained from calf thymus and HMG-E is 62 when measured with anti-HMG-1 and 72 when measured with anti-HMG-E, *i.e.* it has a reciprocal value of 1.15. Using the formula (18) 100(anti-X *versus* Y − anti-Y *versus* X)/½(anti-X *versus* Y + anti-Y *versus* X), it can be calculated that HMG-1 from calf thymus and HMG-E systems deviate from reciprocity by

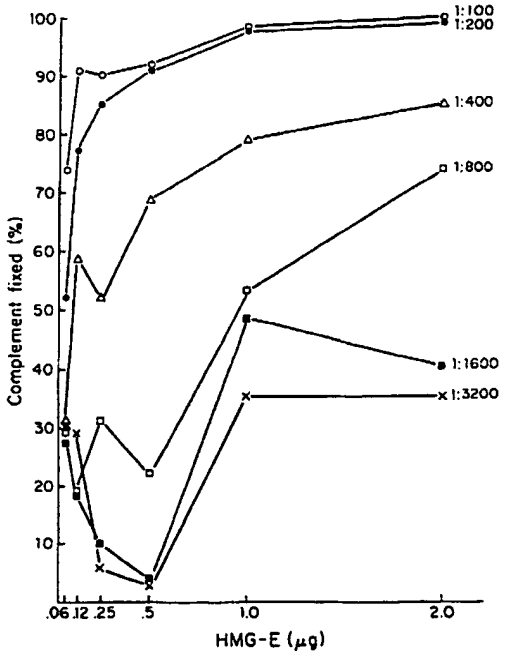

FIG. 2. Complement fixation curves of anti-HMG-E tested with HMG-E from duck erythrocytes. Sera have been tested at the following dilutions: 1:100 (○); 1:200 (●); 1:400 (△); 1:800 (□); 1:1600 (■) and 1:3200 (×).

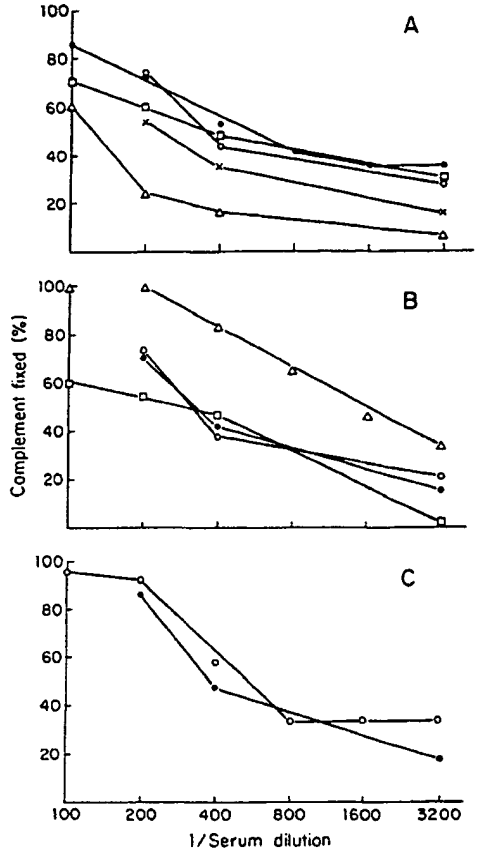

FIG. 3. *A*, determination of index of dissimilarity between various HMG proteins. Plot of the maximal complement fixation at various dilutions of anti-HMG-1 from calf thymus with various HMG proteins. ●, HMG-1 calf thymus; ○, HMG-1 mouse liver; □, HMG-1 duck erythrocytes; ×, HMG-2 calf thymus; △, HMG-E duck erythrocytes. Each *curve* represents the average of at least 3 different experiments. *B*, determination of index of dissimilarity between various HMG proteins. In this experiment, the plot of maximal complement fixation at various dilutions of anti-HMG-E from duck erythrocytes with various HMG proteins is shown. The symbols for the various HMG antigens are the same as in *A*. *C*, determination of index of dissimilarity between HMG-1 from mouse liver and HMG-1 from calf thymus. Plots of the maximal complement fixation are obtained with anti-HMG-1 from mouse liver with the two antigens. Symbols are the same as for *A*.

TABLE III

Immunological distance between HMG proteins

Antigen	Antibody		
	Anti-HMG-1 calf thymus	Anti-HMG-1 mouse liver	Anti-HMG-E duck erythrocytes
HMG-1 calf thymus	0	15	72
HMG-1 mouse liver	15	0	72
HMG-1 erythrocytes	15	NT[a]	79
HMG-2 calf thymus	41	NT[a]	NT[a]
HMG-E erythrocytes	62	NT[a]	0

[a] NT, not tested.

TABLE IV

Sequence differences between HMG-1 from calf thymus and related HMG proteins

Protein	Immunological distance	Sequence difference	Number of substitutions along polypeptide chain[a]
		%	
HMG-1 mouse liver	15	3	7
HMG-1 erythrocytes	15	3	7
HMG-E	67	13	28
HMG-2 calf thymus	40	8	18

[a] It is assumed that HMG-1 has 220 amino acid residues. This assumption is based on a calculated average molecular weight of the amino acids present in HMG-1, which is 122, and a molecular weight of 26,800 determined by several techniques (see Ref. 14).

14.3%. By comparison, mammalian albumins give a reciprocity value of 6.5% and lysozymes, 33%.

It has been reported that in several systems, the immunological distance equals 5 times the percentage sequence difference between related proteins (12, 13). Assuming that this relation holds true for HMG proteins, the percentage sequence difference between the proteins investigated is tabulated in Table IV.

It can be seen that the sequence differences between HMG-1 derived from different species is about 3%. Assuming that HMG-1 has approximately 220 amino acid residues (the footnote in Table IV explains the basis of this assumption), it is possible to estimate that the HMG-1 proteins differ from each other in at least seven distinct loci along the polypeptide chain. This is a minimal estimate since differences in immunosilent regions would not be detected. HMG-E seems to be significantly different from HMG-1. The two proteins differ from each other at about 29 loci in their polypeptide chain.

DISCUSSION

It is currently thought that components present in the so-called nonhistone chromosomal proteins may be involved in aspects of gene regulation. Presently, the proteins belonging to the high mobility group (HMG) proteins are the only nonhistone chromosomal components which can be isolated in good yield and pure form. Determination of the species specificity of these proteins may be of aid in elucidating their role in cellular processes.

In the present study, the sequence similarity of protein HMG-1 purified from three different sources is estimated using serological techniques. It has been previously demonstrated that these techniques provide a simple and rapid way to assess sequence differences between related proteins.

Amino acid analyses and electrophoretic studies suggested that HMG-1 proteins derived from various sources are similar proteins (4). By these criteria, HMG-1 derived from mouse liver is also similar to other HMG-1 proteins. Serological analysis reveals that the sequence differences among HMG-1 derived from calf thymus, mouse liver, and duck erythrocytes are about 3%. Using the same experimental approach, it was found that on the average, the sequence difference between H1 subfractions within a tissue are approximately 10% (13). The sequence difference between H1 obtained from calf thymus and *Drosophila* is 27% (19). Clearly, the HMG-1 display a species specificity significantly lower than this histone. Assuming that HMG-1 protein contains about 220 amino acids, the 3% sequence difference corresponds to substitutions at seven different loci along the polypeptide chain. This can be compared to histones H4 and H3 where sequence data indicate about three substitutions out of 102 residues for H4 and four substitutions out of 135 residues for H3 (20). Thus, the HMG-1 proteins should be considered as proteins whose primary sequence has been conserved during evolution.

The duck erythrocyte-specific HMG-E is clearly different from the HMG-1 proteins. The percentage sequence difference between the two proteins is 13%, corresponding to 29 position differences in amino acid sequence. It has been suggested that HMG-E corresponds to HMG-2 derived from calf thymus (7). Indeed, the two proteins have similar mobilities in acetic acid/urea gels. In the electrophoretic system used here, however, HMG-E clearly migrates in a position different from HMG-2. We have previously noted that the peptide maps of HMG-E and HMG-2 suggest significant differences between the two proteins (4). Serological analyses further support this notion. In a previous study, it was reported that the sequence differences between calf thymus HMG-1 and calf thymus HMG-2 are 6% (14). In the present study, using a new set of antibodies and antigens, the sequence differences between the two proteins appears to be 8% (Tables III and IV).

Because of the widespread occurrence of HMG proteins and the estimation that there are about 1×10^6 HMG molecules/nucleus, it has been suggested (7) that HMG proteins have a structural rather than regulatory function in the nucleus. The present findings that the sequence of HMG-1 is highly conserved further supports this notion. It would appear that their structural role must have stringent sequence requirements. The analogies to the role of histones are obvious.

We feel that the preparation of specific antibodies against purified nonhistone chromosomal proteins such as HMG proteins is an important preliminary step for a fine localization of proteins in chromatin and chromosomes. The importance of serological techniques for the elucidation of chromatin structure has been reviewed (21, 22) and these techniques appear to be fundamental complement to the biochemical approach. The fact that antisera elicited by HMG-1 purified from calf thymus should cross-react with proteins derived from different sources suggests that one type of antisera can be used as a "universal" reagent to study the *in situ* organization of the proteins in a variety of experimental systems. The advantages of such a situation in the study of chromosomal components have already been pointed out (19, 22). Antibodies can be used not only for the localization of the respective antigen but also employed for affinity chromatography purification of those regions of the chromatin where these proteins are specifically found.

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